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**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Nucleic acid sequences that can be used as primers and probes in the amplification and detection of SARS Coronavirus

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**Nucleic acid sequences that can be used as primers and probes in the amplification and detection of SARS Coronavirus**

The present invention is related to nucleic acid sequences that can be used in the field of virus diagnostics, more specifically the diagnosis of infections with a novel human coronavirus causing Severe Acute Respiratory Syndrome (SARS).

An outbreak of atypical pneumonia, referred to as SARS, was first identified in late 2002 in Guangdong Province, China (Marra *et al.* (1) or Rota *et al.* (2)). After similar cases were detected in patients in Hong Kong, Vietnam, and Canada during February and March 2003, the World Health Organization (WHO) issued a global alert for the illness. By May 22nd, 2003, over 8000 SARS cases and 682 SARS-related deaths were reported to WHO from over 30 countries around the world.

Most of these cases were related to exposure to SARS patients in residential or healthcare settings. The incubation period for the disease usually ranges from 2 to 7 days. Infection is usually characterized by fever and flu-like symptoms, which are followed a few days later by a dry, non-productive cough, and shortness of breath. Death from progressive respiratory failure occurs in about 3% to nearly 10% of cases (3–7).

Conventional diagnostic kits could authorize the detection of antibodies produced in response to the SARS coronavirus infection. Different types of antibodies (IgM and IgG) appear and change in level during the course of infection. They can be undetectable at the early stage of infection. Immunoglobulin G usually remains detectable after resolution of the illness.

The following test formats are being developed, but are not commercially available yet:
- Enzyme Linked ImmunoSorbant Assay (ELISA): a test detecting a mixture of IgM and IgG antibodies in the serum of SARS patients yields positive results reliably at around day 21 after the onset of illness, and
- Immunofluorescence Assay (IFA): a test detecting IgM antibodies in serum of SARS patients yields positive results after about day 10 of illness; this test format is also used to test for IgG; this is a reliable test requiring the use of fixed SARS virus infected cell cultures, studied under an immunofluorescence microscope.

Positive antibody test results indicate a previous infection with SARS-Coronavirus. Seroconversion from negative to positive or a four-fold rise in antibody titre from acute to convalescent serum indicates recent infection.

With negative antibody test results, no detection of antibody after 21 days from onset of illness seems to indicate that no infection with SARS-Coronavirus took place.

Clearly, the current generation of SARS antibody tests, which use the immunofluorescence and ELISA techniques, have a sensitivity problem. Immunofluorescence can only detect the antibodies after a patient has carried the disease for at least 10 days, while for ELISA, about 20 days are needed. During that time, the patient may have spread the virus to other people. In that context, the tests are mainly useful after hospitalisation, for confirming whether a patient has SARS. That is the reason why these tests are not very useful for infectious disease control. Thus the need exists for a bedside test, a quick on-the-spot test which allows the detection (from secretions) at an early stage of the disease or the detection of early antibodies, say from day five (of infection) onwards.

Another conventional detection method is constituted by cell cultures. Virus in specimens (such as respiratory secretions, blood or stool) from SARS patients can also be detected by inoculating cell cultures and growing the virus. Once isolated, the virus must be identified as the SARS virus with further tests. Cell culture is a very demanding test, but currently (with the exception of animal trials) the only means to show the existence of a live virus.

Positive cell culture results indicate the presence of live SARS-Coronavirus in the sample tested.

Negative cell culture results do not exclude SARS.

Moreover these two conventional techniques (detection of antibodies and cell culture) require a laboratory and trained staff as well as time to carry out the test. These tests are good and robust in scientific laboratories but they are not rapid enough to have an efficient diagnosis in due time.

Whereas conventional virus diagnosis has been based predominantly on the detection of viral antigens or specific antibodies thereto, in recent years attention has shifted towards methods for the direct and rapid detection of the genome of viruses or nucleic acid sequences derived thereof, both RNA and DNA. In this respect, the very short time-to-result is a crucial factor to opt for nucleic acid detection. These methods are usually based on nucleic acid hybridization. Nucleic acid hybridization is based on the ability of two strands of nucleic acid containing complementary sequences to anneal to each other under the appropriate conditions,

thus forming a double stranded structure. When the complementary strand is labeled, the label can be detected and is indicative for the presence of the target sequence. Especially in combination with methods for the amplification of nucleic acid sequences these methods have become an important tool in viral diagnosis.

Nucleic acid amplification techniques are especially useful as an additional technique in cases where serological methods give doubtful results or in cases where there may be a considerable time period between infection and the development of antibodies to the virus. With HIV for example, seroconversion usually can occur some 3-6 weeks after exposure to the virus. Thus, whereas no antibodies will be detected with conventional immunoassays, proviral DNA or circulating viral RNA may already be detectable. Also in monitoring antiviral therapy, methods based on nucleic acid amplification have several advantages over serological methods. Especially quantitative amplification methods provide a powerful tool in assessing the changes in the amount of virus present before and during therapy.

The choice of the oligonucleotides to be used as primers and probes in the amplification and detection of nucleic acid sequences is critical for the sensitivity and specificity of the assay. The sequence to be amplified is usually only present in a sample (for example a blood sample obtained from a patient suspected of having a viral infection) in minute amounts. The primers should be sufficiently complementary to the target sequence to allow efficient amplification of the viral nucleic acid present in the sample. If the primers do not anneal properly (due to mispairing of the bases on the nucleotides in both strands) to the target sequence, amplification is seriously hampered. This will affect the sensitivity of the assay and may result in false negative test results. Due to the heterogeneity of viral genomes false negative test results may be obtained if the primers and probes are capable of recognizing sequences present in only part of the variants of the virus.

Various techniques for amplifying nucleic acid are known in the art. One example of a technique for the amplification of a DNA target segment is the so-called "polymerase chain reaction" (PCR). With the PCR technique the copy number of a particular target segment is increased exponentially with a number of cycles. A pair of primers is used and in each cycle a DNA primer is annealed to the 3' side of each of the two strands of the double stranded DNA-target sequence. The primers are extended with a DNA polymerase in the presence of the various mononucleotides to generate double stranded DNA again. The strands of the double stranded DNA are separated from each other by thermal denaturation and each strand serves as a template for primer annealing and subsequent elongation in a following cycle. The PCR

method has been described in Saiki *et al.*, Science 230, 135, 1985 and in patents EP-B-0.200.362 and EP-A-0.201.184.

Another technique for the amplification of nucleic acid is the so-called transcription based amplification system (TAS). The TAS method is described in WO-A-88/10315. Transcription based amplification techniques usually comprise treating target nucleic acid with two oligonucleotides one of which comprises a promoter sequence, to generate a template including a functional promoter. Multiple copies of RNA are transcribed from said template and can serve as a basis for further amplification.

An isothermal continuous transcription based amplification method is the so-called NASBA process ("NASBA") as described in EP-B-0.329.822. NASBA includes the use of T7 RNA polymerase to transcribe multiple copies of RNA from a template including a T7 promoter. Other transcription based amplification techniques are described in EP-A-0.408.295. EP-A-0.408.295 is primarily concerned with a two-enzyme transcription based amplification method. Transcription based amplification methods, such as the NASBA method as described in EP-A-0.329.822, are usually employed with a set of oligonucleotides, one of which is provided with a promoter sequence that is recognized by a DNA dependent RNA polymerase such as, for example, T7 RNA polymerase. Several modifications of transcription-based techniques are known in the art. These modifications comprise, for example, the use of blocked oligonucleotides (that may be provided with a promoter sequence). These oligonucleotides are blocked so as to inhibit an extension reaction proceeding there from (US-A-5,554,516). One or more "promoter-primers" (oligonucleotides provided with a promoter sequence) may be used in transcription based amplification techniques, optionally combined with the use of one or more oligonucleotides that are not provided with a promoter sequence. For RNA amplification, a transcription based amplification technique, is a preferred technology.

Amplification using PCR can also be based on an RNA template. The actual PCR needs to be preceded by a reverse transcription step to copy the RNA into DNA (RT-PCR). However, if RT-PCR is used for the detection of viral transcripts, differentiation of mRNA- and DNA-derived PCR products is necessary. DNAse treatment prior to RT-PCR can be employed (Bitsch *et al.* (8); Meyer *et al.* (9)), but sometimes fails to remove contaminating DNA sufficiently (Bitsch *et al.* (8)).

That is the solution advocated by Drosten *et al.* (10) for the detection of the SARS Coronavirus. Thus by performing random amplification with fifteen different PCRs under low-stringency conditions and including, an initial reverse-transcription step to detect RNA viruses, about twenty distinct DNA fragments were obtained and sequenced. Three of the fragments did not match any nucleotide sequence in the database (www.ncbi.nlm.nih.gov:80/BLAST). In a translated BLAST search these fragments showed homology to coronavirus amino acid sequences. Two of the fragments were 300 nucleotides in length and identical in sequence, and the third fragment was 90 nucleotides in length (sequences BNI-1 and BNI-2, respectively, as reported on the Web site of the WHO network on March 25). Detailed sequence analysis revealed that both fragments were located in the open reading frame 1b of coronaviruses. The novel coronavirus isolate was termed FFM-ic (for Frankfurt am Main index case).

Simultaneously, a group at the Centers for Disease Control and Prevention (CDC), Atlanta, USA identified a 400-nucleotide coronavirus fragment (Urbani isolate, reported on the Web site of the WHO network on March 24). This sequence did not show an overlap with the BNI-1 and BNI-2 sequences (Ksiazek *et al.* (13)).

When primers specifically targeting the BNI-1 fragment and the CDC fragment were used in a long-range PCR protocol, a region extending from the CDC fragment to the BNI-1 fragment was amplified. The obtained fragment had the expected length of 3 kb. It was sequenced from both ends, and the sequences were found to be identical to the CDC and BNI-1 sequences, respectively, demonstrating that the two sequences were derived from a contiguous RNA molecule and, thus, from the same virus. Since the same virus was isolated from two independent cases of SARS, and since there was serologic evidence of an acute infection with this virus in the underlying patients, it was considered that the coronavirus might have a role in causing SARS. Specific diagnostic assays based on RT-PCR were therefore established for the detection of the pathogen.

However, PCR and RT-PCR also have problems. Drosten *et al.* (10) established a diagnostic method for SARS that makes use of a nested set of primers designed within the BNI-1 fragment. The outer set of primers detected the virus in clinical specimens from the index patient and different patients, who also had clinical signs of SARS. Additional specimens were positive on nested RT-PCR. To have a practical and quantitative test, a real-time RT-PCR with a 5'-nuclease probe was established. After optimization with the use of quantified RNA transcribed in vitro, the assay reliably detected 10 copies of RNA per reaction, corresponding to

830 RNA molecules per millilitre of specimen. The sensitivities of nested and real-time PCR were equivalent.

The specificity of the PCR with outer primers of the nested PCR, and of the real-time PCR was tested with the use of RNA purified from cultures of bovine coronavirus, avian infectious coronavirus, porcine transmissible gastroenteritis coronavirus and human coronaviruses 229E and OC43. None of the PCR assays cross-reacted with these viruses - a finding that is consistent with their considerable genetic differences from the novel coronavirus. Quantification of the viral RNA concentration in clinical specimens from the index patient and Contact 1 by real-time PCR revealed that the highest concentration - as high as 100 million copies per millilitre - was present in sputum. After enrichment of virus by ultra centrifugation, viral RNA was also detected in the serum of the index patient, indicating the presence of low-level viremia during symptomatic disease. Both the index patient and Contact 1 had viral RNA in stool samples obtained during late convalescence, suggesting that virus may be shed in faeces for prolonged periods of time. To investigate whether the novel coronavirus was prevalent in patients in Germany who had gastrointestinal symptoms, a collection of fifty-four stored stool samples was tested with the use of the real-time RT-PCR assay. None of the samples tested positive.

The established PCR assays have also been used to test respiratory samples from German patients with symptoms and a travel history compatible with SARS. So far, sixty-seven samples from fifty-five patients have been tested. One patient fulfilling the WHO criteria for probable SARS was coronavirus-positive on PCR. PCR protocols, as well as positive control material, have been made available to laboratories worldwide.

Until standardized reagents for virus and antibodies detection are available and methods have been adequately field tested, SARS diagnosis remains based on the clinical and epidemiological findings: acute febrile illness with respiratory symptoms not attributed to another cause and a history of exposure to a suspect or probable case of SARS or their respiratory secretions and other bodily fluids. Those requirements are reflected in the current WHO case definitions for suspect or probable SARS.

Researchers in several countries are working towards developing fast and accurate laboratory diagnostic tests for the SARS-Coronavirus. Molecular tests based on PCR amplification are one of the faster and more accurate tests that could achieve this goal.

Polymerase chain reaction (PCR) can detect genetic material of the SARS-Coronavirus in various specimens (blood, stool, respiratory secretions or body tissues). Same primers, which

are the key pieces for a PCR test, have been made publicly available by WHO network laboratories on the WHO web site. A ready-to-use PCR test kit containing primers and a positive and negative control has been developed. Testing of the kit by network members is expected to quickly yield the data needed to assess the test's performance, in comparison with primers developed by other WHO network laboratories and in correlation with clinical and epidemiological data. Principally, existing PCR tests are very specific but lack sensitivity. This means that negative tests cannot rule out the presence of the SARS virus in patients. Furthermore, contamination of samples in laboratories in the absence of laboratory quality control can lead to false positive results.

Positive PCR results, with the necessary quality control procedures in place, are very specific and mean that there is genetic material (RNA) of the SARS-Coronavirus in the sample. This does not mean that there is live virus present, or that it is present in a quantity large enough to infect another person.

Negative PCR results do not exclude SARS. SARS-Coronavirus PCR can be negative for the following reasons:

- the patient is not infected with the SARS coronavirus; the illness is due to another infectious agent (virus, bacterium, fungus) or a non-infectious cause,
- the test results are incorrect ("false-negative"). Current tests need to be further developed to improve sensitivity,
- specimens were not collected at a time when the virus or its genetic material was present. The virus and its genetic material may be present for a brief period only, depending on the type of specimen tested,
- specimens were not properly handled prior to nucleic acid extraction and nucleic acids have become deteriorated.

The use of the oligonucleotides according to the invention is not limited to any particular amplification technique or any particular modification thereof, however these oligonucleotides permit amplification of RNA by using a transcription-based amplification technique, preferably the NASBA. Our primers sets are very efficient compared to the RT-PCR primers. In contrast to RT-PCR, NASBA, which is based on RNA transcription by T7 RNA polymerase (Kievits *et al.* (11); Compton (12)), does not need differentiation between RNA- and DNA-derived amplification products since it uses RNA as its principal target. RT-PCR for SARS have a too low sensitivity, often nested PCR protocol needed to achieve satisfying

sensitivity. The nested protocol is then riskful in terms of contamination, i.e. falses positives. NASBA can achieve sensitivity comparable to nested protocol in a "one-step" amplification avoiding risk for contamination. It is the purpose of this invention to provide reagents and methods to realize kits for diagnosing SARS disease earlier than former kits.

The oligonucleotides of the present invention can likewise be used in quantitative amplification methods. An example if such quantitative method is described in EP-A-0.525.882.

The term "oligonucleotide" as used herein refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. Such oligonucleotides may be used as primers and probes.

Of course, based on the sequences of the oligonucleotides of the present invention, analogues of oligonucleotides can also be prepared. Such analogues may constitute alternative structures such as "PNA" (molecules with a peptide-like backbone instead of the phosphate sugar backbone of normal nucleic acid) or the like. It is evident that these alternative structures, representing the sequences of the present invention are likewise part of the present invention.

The term "primer" as used herein refers to an oligonucleotide either naturally occurring (e.g. as a restriction fragment) or produced synthetically, which is capable of acting as a point of initiation of synthesis of a primer extension product which is complementary to a nucleic acid strand (template or target sequence) when placed under suitable conditions (e.g. buffer, salt, temperature and pH) in the presence of nucleotides and an agent for nucleic acid polymerization, such as DNA dependent or RNA dependent polymerase. A primer must be sufficiently long to prime the synthesis of extension products in the presence of an agent for polymerization. A typical primer contains at least about 10 nucleotides in length of a sequence substantially complementary or homologous to the target sequence, but somewhat longer primers are preferred. Usually primers contain about 15-28 nucleotides, preferably 20-26 nucleotides, but longer primers may also be employed, especially when the primers contain additional sequences such as a promoter sequence for a particular polymerase.

Normally a set of primers will consist of at least two primers, one "upstream" primer and one "downstream" primer, which together define the amplicon (the sequence that will be amplified using said primers).

Primarily for the use in transcription based amplification techniques, the oligonucleotides according to the invention may also be linked to a promoter sequence. The term "promoter sequence" defines a region of a nucleic acid sequence that is specifically

recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription by which an RNA transcript is produced. In principle any promoter sequence may be employed for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Known and useful promoters are those that are recognized by certain bacteriophage RNA polymerases such as bacteriophage T3, T7 or SP6. Oligonucleotides linked to a promoter sequence are commonly referred to as "promoter primers". Their function as a primer, e.g. the starting point for an elongation reaction, however, may be blocked, as already mentioned above, or absent in some embodiments of transcription based amplification reactions.

A pair of oligonucleotides, according to the present invention, is used as a set in the amplification of a target sequence located within the region located in the replicase 1ab gene of the genome of SARS Coronavirus, said pair consisting of:

- a first oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 1: AAGATGTTTA AACTGGTCAC CTGGTGGAGG TTTTGCATTA ACTCTGGTGA ATTCTGTGTT ATTTTCAGTG TCAACATAAC CAGTCGGTAC AGCTACTAAG TTAACACCTG TAGAAAATCC TAGCTGGAGA GGTA, or the complementary sequence thereof, and
- a second oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 2: ATGAATTACC AAGTCAATGG TTACCCTAAT ATGTTTATCA CCCGCGAAGA AGCTATTCGT CACGTTCGTG CGTGGATTGG CTTTGATGT, or the complementary sequence thereof.

In a preferred embodiment, the pair of oligonucleotides consists essentially of:

- a first oligonucleotide comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group consisting of:
  - SEQ ID 3: AAGATGTTTA AACTGGTCAC CTGGTGGA,
  - SEQ ID 4: AACATAACCA GTCGGTACAG CTACTA,
  - SEQ ID 5: AGAAAATCCT AGCTGGAGAG GTA,

  or the complementary sequence thereof, and
- a second oligonucleotide comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group consisting of:
  - SEQ ID 6: ATGAATTACC AAGTCAATGG TTAC,
  - SED ID 7: GAAGCTATTC GTCACGTTCG,

- SEQ ID 8: TGCGTGGATT GGCTTTGATG T,

or the complementary sequence thereof.

It is understood that oligonucleotides consisting of the sequences of the present invention may contain minor deletions, additions and/or substitutions of nucleic acid bases, to the extent that such alterations do not negatively affect the yield or product obtained to a significant degree. Where oligonucleotides according to the present invention are used as probes, the alterations should not result in lowering the hybridization efficiency of the probe.

For example, in case of transcription based amplification techniques, wherein one or more of the primers may be provided with a promoter sequence, the introduction of a purine-rich (= G or A) hybridizing sequence, just after the promoter sequence may have positive effects on the transcription (when there are C's and T's abortive transcription may occur). If no such sequence is available in the target nucleic acid a purine-rich sequence can be inserted in the oligonucleotide just following the last three G residues of the promoter sequence.

The sequences of the present invention are reflected as DNA sequences. The RNA equivalents of these sequences are likewise part of the present invention.

The sequences SEQ ID 9-11 actually comprise the sequence as reflected by SEQ ID 3-5. In SEQ ID 9-11, the sequences of SEQ ID 3-5 are operably linked to a promoter sequence (the T7 promoter sequence). This makes the sequences especially suitable for use as downstream primer in a transcription based amplification technique such as NASBA. A first oligonucleotide particularly efficient for such an amplification technique, consists essentially of the sequence:

- SEQ ID 9: *aattctaata cgactcacta tagggAAGAT* GTTTAAACTG GTCACCTGGT GGA,
- SEQ ID 10: *aattctaata cgactcacta tagggAACAT* AACCAGTCGG TACAGCTACT A, or
- SEQ ID 11: *aattctaata cgactcacta tagggAGAAA* ATCCTAGCTG GAGAGGTA,

or the complementary sequence thereof.

One of the oligonucleotides may serve as an "upstream oligonucleotide", i.e., an upstream-primer, while the second oligonucleotide serves as a "downstream oligonucleotide", i.e. downstream primer, in the amplification reaction. The location on the SARS-genome (or the sequence complementary thereto) to which both oligonucleotides comprised in such a pair according to the invention can anneal, will together define the sequence of the nucleic acid that

is amplified. The amplified sequence is located between the "primer-binding sites" within the SARS genome. It has been found that, by using a pair of oligonucleotides according to the invention in an amplification reaction, accurate and reliable amplification of nucleic acid of SARS can be achieved.

A most preferred pair of oligonucleotides according to the invention consist of a first primer comprising the sequence of SEQ ID NO 4 or 10 and a second primer with the sequence of SEQ ID NO 7. For use in a transcription based amplification method, especially the NASBA amplification, the oligonucleotide with SEQ ID NO 10 is preferred, in combination with an oligonucleotide with the sequence of SEQ ID NO 7.

Part of the oligonucleotides according to the invention are particularly suitable for use as a probe in the detection of nucleic acid amplified with a pair of oligonucleotides according to the invention. When used as a probe, said oligonucleotides may be provided with a detectable label. Oligonucleotides according to the invention that are especially suitable as a probe consist of sequences of 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 12: AGAGGGCTGT CATGCAACTA GAGATGCTGT GGGTACTAACC, or the complementary sequence thereof, provided with a detectable label.

A most preferred oligonucleotide in this respect is an oligonucleotide with a sequence as depicted in SEQ ID 14: CTGTCATGCA ACTAGAGATG CTGT.

Various labeling moieties are known in the art. Said moiety may, for example, either be a radioactive compound, a detectable enzyme (e.g. horse radish peroxidase (HRP)), a hapten like biotin, or any other moiety capable of generating a detectable signal such as a colorimetric, fluorescent, chemiluminescent or electrochemiluminescent signal.

Hybrids between oligonucleotides according to the invention and (amplified) target nucleic acid may also be detected by other methods known to those skilled in the art.

Evidently methods for amplification of nucleic acid, like the ones that have been mentioned above, using the oligonucleotides according to the present invention are also part of the invention.

According to the invention, the more efficient probe is the one, which is constituted by a molecular beacon, preferably consisting of:

SEQ ID 13: [6-FAM]-ccatgggCTGTCATGCAACTAGAGATGCTGTcccatgg-[DabSyl].

The sequence in capital letters refers to the probe itself according to SEQ ID 14, i.e. where the hybridisation between said probe and a part of the amplified nucleic acid takes place. Downstream and upstream extensions, in small letters, are sequences called here 7-nucleotide-

long arm sequences that are able to hybridise one to the other. These arm sequences relate to linkers between the probe and on the one hand a fluorophore (6-FAM) and on the other hand quencher 4-(4'-dimethylaminophenylazo) benzoic acid (DabSyl). An ideal fluorophore-quencher pair is completely unable to fluoresce when the two components are in close proximity, but as soon as the loop of the molecular beacon is conformationally changed due to hybridization of the probe to the amplified nucleic acid, the distance between the fluorophore and the quencher is sufficiently enlarged to permit emission of fluorescence.

Other fluorescent reporters are possible like 5' fluorescein, 5' TET, 5' HEX, 5' FAM, 5' TAMRA, 5' ROX, 5' Texas Red, 5' Oregon Green, 5' Cy3 or 5' Cy5. In the same way other quenchers, such as 3' Black Hole Quencher-1 or 3' Black Hole Quencher-2, can be used.

The present invention further provides test kits for the amplification and detection of SARS nucleic acid. The use of said test kits enables accurate and sensitive screening of samples suspected of containing SARS derived nucleic acid. Such test kits may contain a pair of oligonucleotides according to the invention and optionally also an oligonucleotide according to the invention that can be used as a probe for the detection of the amplified material. Furthermore the test-kit may contain suitable amplification reagents. These reagents are for example the suitable enzymes for carrying out the amplification reaction. A kit, adapted for use with NASBA, for example may contain suitable amounts of reverse transcriptase, RNase H and T7 RNA polymerase. Said enzymes may be present in the kit in a buffered solution but can likewise be provided as a lyophilized composition, for example, a lyophilized spherical particle. Such lyophilized particles have been disclosed in PCT/EP95/01268. The kit may further be furnished with buffer compositions, suitable for carrying out an amplification reaction. Said buffers may be optimized for the particular amplification technique for which the kit is intended as well as for use with the particular oligonucleotides that are provided with the kit. In transcription-based amplification techniques, such as NASBA, said buffers might contain, for example, DMSO, which enhances the amplification reaction (as is disclosed in PCT/US90/04733).

Furthermore the kit may be provided with an internal control as a check on the amplification procedure and to prevent the occurrence of false negative test results due to failures in the amplification procedure. The use of internal controls in transcription based amplification techniques is described in PCT/EP93/02248. An optimal control sequence is selected in such a way that it will not compete with the target nucleic acid in the amplification

reaction. Kits may also contain reagents for the isolation of nucleic acid from biological specimens prior to amplification. A suitable method for the isolation of nucleic acid is disclosed in EP-A-0.389.063.

Virus propagation and isolation from in-vitro cultures:

A variety of clinical specimens (blood, serum, material from oropharyngeal swabs or washings, material from nasopharyngeal swabs, and tissues of major organs collected at autopsy) was inoculated onto a number of continuous cell lines, including Vero E6, NCIH292, MDCK, LLC-MK2 and B95-8 cells. Two cell lines, Vero E6 cells and NCI-H292 cells, inoculated with oropharyngeal specimens from patients with SARS initially showed cytopathic effect. A rhinovirus was isolated from the inoculated NCI-H292 cells, but further study suggested that this virus was not associated with patients with SARS. Examination of cytopathic-effect–positive VeroE6 cells by thin-section electron microscopy revealed characteristic coronavirus particles within the cisternae of the rough endoplasmic reticulum and in vesicles. Extracellular particles were found in large clusters and adhering to the surface of the plasma membrane.

Vero E6 cells have now become the standard cell line for the isolation and propagation of the SARS related human Coronavirus (Marra *et al.* (1), Drosten *et al.* (10), Ksiazek *et al.* (13)).

Identification of specific nucleic acid fragments of the SARS related human Coronavirus:

This identification has already been disclosed previously in the disclosure according to a publication (Drosten *et al.* (10)).

A group at the National Microbiology Laboratory in Canada also obtained a SARS genomic sequence (Genbank Accession AY274119.3) from an isolate referred to as Tor2. The latter sequence is essentially identical to the one that was released independently by the CDC (GenBank accession number AY278741). Additional bases in the Tor2 sequence that correspond to the 3′ (encoded) polyA tail were reported. Eight base differences between the two sequences could represent sequencing errors, PCR artefacts or mutable sites in the genome.(Marra *et al.* (6)). The genome of SARS-Coronavirus is a 29,727-nucleotides polyadenylated RNA, and 41% of the residues are G or C The genomic organization is typical of coronaviruses, with the characteristic gene order. The SARS-Coronavirus replicase gene, which comprises approximately two-thirds of the genome, is predicted to encode two polyproteins (encoded by ORF1a and ORF1b) that undergo co-translational proteolytic

processing. There are four open reading frames (ORFs) downstream of replicase that are predicted to encode the structural proteins S, E, M, and N, which are common to all known coronaviruses. The hemagglutinin-esterase (HE) gene, which is present between ORF1b and S in group 2 and some group 3 coronaviruses, was not found. (Rota *et al.* (7)). This is in agreement with the Electron microscopic observations of virus particles lacking the HE surface projections.

Phylogenetic analyses of the sequence of SARS-Coronavirus indicate that SARS-Coronavirus is not closely related to any of the previously characterized coronaviruses and forms a distinct group within the genus *Coronavirus*. The SARS-Coronavirus is approximately equidistant from all previously characterized coronaviruses, just as the existing groups are from one another. No evidence for recombination was detected when the predicted protein sequences were analyzed using SimPlot in comparison to sequences of known Coronaviruses.

The coronavirus subgenomic mRNAs are synthesized through a discontinuous transcription process, the mechanism of which has not been unequivocally established. The favoured model for production of subgenomic mRNAs of coronaviruses proposes that discontinuous transcription occurs during synthesis of the negative strand (Sawicki G. S. *et al.* (14)). The positions of the transcription regulating sequences (TRS) in the genome of SARS-Coronavirus predict that subgenomic mRNAs of 8.3, 4.5, 3.4, 2.5, 2.0, and 1.7 kb, not including the poly(A) tail, should be produced.

At least five subgenomic mRNAs were indeed detected by Northern hybridization of RNA from SARS-Coronavirus-infected cells, using a probe derived from the 3'-untranslated region. Full-length genomic RNA was not detected, likely because it is the least prevalent viral RNA in infected cells (Lai *et al.* (15)). The predicted 2.0 kb transcript was also not detected, suggesting that the consensus TRS at nucleotides 27,771-27,778 is not used or that it is a low-abundance mRNA (Rota *et al.* (2)).

The invention will now be described further, by way of examples, with reference to the accompanying drawings, in which:

- Figure 1: Amplification curves of 4 different primer pairs tested on a dilution series of SARS Coronavirus in vitro RNA, and
- Figure 2: Amplification curves of the selected primer pair (P1.1/P2.2), testing the analytical sensitivity of the SARS CoV NASBA.

**Examples:**

**Materials and Methods:**

1. SARS Coronavirus in vitro RNA:

RNA encompassing 190 nucleotides of the SARS Coronavirus genomic sequence encoding the viral polymerase protein was synthesized in vitro by transcription of a cloned fragment of the viral replicase gene, essentially as described in Drosten *et al.* (10). The concentration of the in vitro generated RNA was determined by OD (260 nm) measurement and appropriate serial dilutions in water were stored at -70°C until further use.

2. Oligonucleotides used for amplification and detection:

Primers and a molecular beacon detection probe (Tyagi, S. and Kramer, F.R. (16)) were derived from the sequence of the open reading frame (ORF) 1b of the SARS Coronavirus replicase gene and realized as HPLC-purified oligonucleotides. Sequences and polarities of the oligonucleotides used in the amplification and of the molecular beacon probe used for specific detection in real-time, are shown in Table 1.

| Sequence number | Primer/ Probe | Location |
|---|---|---|
| SEQ ID 4 | Downstream primer P1.1 | 18319-18344 |
| SEQ ID 3 | Downstream primer P1.2 | 18283-18305 |
| SEQ ID 6 | Upstream primer P2.1 | 18153-18176 |
| SEQ ID 7 | Upstream primer P2.2 | 18201-18220 |
| SEQ ID 13 | Probe MB8500 | 18246-18271 |

Table 1: Primers and molecular beacon probe for the amplification and detection in real-time of a region located in the replicase 1ab gene of SARS Coronavirus.

For the P1 primers (P1.1, P1.2) the T7 polymerase promoter sequence is depicted in small characters. For the molecular beacon (MB8500) the arm sequences at the 5'-end and at the 3'-end are depicted in small characters. Fluorophore 6-FAM is covalently linked to the 5'-end of the molecular beacon; the quenching moiety DabSyl is covalently linked to the 3'-end of the molecular beacon. Coordinates for the location of the hybridising segments of the primers

and the molecular beacon probe are derived from the complete genome sequence of SARS coronavirus TOR2 (EMBL Accession number AY274119).

3. NASBA amplification with real-time detection:

RNA amplifications were performed using the NASBA amplification technology. To set up a NASBA amplification reaction, NucliSens Basic Kit Amplification Reagents (bioMérieux b.v., The Netherlands) were used. A premix was generated by reconstituting a Reagent Sphere in a mixture of 80 µl Reagent Sphere Diluent, 18 µl KCl stock solution and 12 µl NASBA Water. Subsequently, 10 µl of a mix containing two primers for amplification and a molecular beacon probe for real-time detection, were added, resulting in 2 x reaction buffer. Ten µl of this reaction buffer was added to 5 µl nucleic acid solution and incubated during 5 minutes at 65°C. Then the reaction tubes were incubated at 41°C during 5 minutes. Meanwhile, an Enzyme Sphere was reconstituted in 55 µl Enzyme Diluent and 5 µl of the resulting enzyme mix was added into each reaction tube. After addition of the enzyme mix, tubes were mixed by gentle tapping, centrifuged briefly to bring the contents to the bottom of the tubes and finally incubated at 41°C during 90 minutes in a NucliSens EasyQ Analyzer. Fluorescence in the individual reaction tubes was monitored over time and measured at regular intervals of 45 seconds.

**Example 1: Selection of primer pair for the amplification of SARS CoV RNA.**

To select a well performing primer pair for the amplification of SARS Coronavirus RNA using NASBA, two upstream primers, designated P2.1 and P2.2, and two downstream primers, designated P1.1 and P1.2, were derived from the sequence of the open reading frame (ORF) 1b of the SARS Coronavirus replicase gene. Consequently, four different primer pairs could be formed, each consisting of a combination of one of the upstream primers with one of the downstream primers. With each of these primer pairs a standard dilution series of in vitro generated SARS Coronavirus RNA was evaluated.

Results are summarized in Figures 1-4 and show that all primer pairs revealed a positive result for the different amounts of in vitro RNA that were tested. However, analysis of the moment at which the different reactions start to reveal fluorescence signals that are well above the background signal showed that primer combination P2.2/P1.1 has strikingly lower Time-To-Positivity (TTP) values as the other primer combinations. Therefore, oligonucleotide

primers P1.1 and P2.2 were selected as the most ideal primer pair for the amplification of SARS Coronavirus RNA.

**Example 2: Analytical sensitivity of the selected primer pair.**

To determine the analytical sensitivity of the selected primer pair (P1.1/P2.2) a dilution series of SARS Coronavirus in vitro RNA was tested with the selected primer pair. The RNA dilution series was made in NASBA water from a $1x10^5$ copies/µl RNA stock solution. Results are summarized in Figure 5 and show a very good analytical sensitivity with positive results down to 2.5 copies of in vitro RNA in de amplification, while the negative control remained negative.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: bioMérieux B.V.
(B) STREET: Postbus 84
(C) CITY: Boxtel
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 5280 AB

(ii) TITLE OF INVENTION: Nucleic acid sequences that can be used as primers and probes in the amplification and detection of SARS.

(iii) NUMBER OF SEQUENCES: 14

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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ATTTTCAGTG TCAACATAAC CAGTCGGTAC AGCTACTAAG TTAACACCTG TAGAAAATCC
TAGCTGGAGA GGTA 134

(2) INFORMATION FOR SEQ ID NO: 2:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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CACGTTCGTG CGTGGATTGG CTTTGATGT 89

(2) INFORMATION FOR SEQ ID NO: 3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AACATAACCA GTCGGTACAG CTACTA 26

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(ii) MOLECULE TYPE: DNA

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(ii) MOLECULE TYPE: cDNA

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(C) STRANDEDNESS: single
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(ii) MOLECULE TYPE: cDNA

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTGTCATGCA ACTAGAGATG CTGT 24

## CLAIMS

1. Pair of oligonucleotides, for use as a set in the amplification of a target sequence located within the region located in the polymerase gene of the genome of SARS Coronavirus, said pair consisting of:

- a first oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 1: AAGATGTTTA AACTGGTCAC CTGGTGGAGG TTTTGCATTA ACTCTGGTGA ATTCTGTGTT ATTTTCAGTG TCAACATAAC CAGTCGGTAC AGCTACTAAG TTAACACCTG TAGAAAATCC TAGCTGGAGA GGTA, or the complementary sequence thereof, and
- a second oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 2: ATGAATTACC AAGTCAATGG TTACCCTAAT ATGTTTATCA CCCGCGAAGA AGCTATTCGT CACGTTCGTG CGTGGATTGG CTTTGATGT, or the complementary sequence thereof.

2. Pair of oligonucleotides, according to claim 1, consisting essentially of:

- a first oligonucleotide comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group consisting of:
  - SEQ ID 3: AAGATGTTTA AACTGGTCAC CTGGTGGA,
  - SEQ ID 4: AACATAACCA GTCGGTACAG CTACTA,
  - SEQ ID 5: AGAAAATCCT AGCTGGAGAG GTA,

  or the complementary sequence thereof, and
- a second oligonucleotide comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group consisting of:
  - SEQ ID 6: ATGAATTACC AAGTCAATGG TTAC,
  - SED ID 7: GAAGCTATTC GTCACGTTCG,
  - SEQ ID 8: TGCGTGGATT GGCTTTGATG T,

  or the complementary sequence thereof.

3. Pair of oligonucleotides, according to claim 1 or 2, wherein the first oligonucleotide is provided with a promoter sequence recognized by a DNA dependent RNA polymerase.

4. Pair of oligonucleotides, according to claim 3, wherein the first oligonucleotide consists essentially of the sequence:

- SEQ ID 9: *aattctaata cgactcacta tagggAAGAT* GTTTAAACTG GTCACCTGGT GGA,
- SEQ ID 10: *aattctaata cgactcacta tagggAACAT* AACCAGTCGG TACAGCTACT A, or
- SEQ ID 11: *aattctaata cgactcacta tagggAGAAA* ATCCTAGCTG GAGAGGTA,

or the complementary sequence thereof.

5. Pair of oligonucleotides, according to any of the claims 1-4, wherein each oligonucleotide being 15-30 nucleotides in length and comprising at least a fragment of 18 nucleotides, and preferably being 18-26 nucleotides in length and comprising at least a fragment of 20 nucleotides.

6. Oligonucleotide, for use as a probe to detect the amplified nucleic acid sequence resulting in the amplification of a target sequence located within the region located in the polymerase gene of the genome of SARS Coronavirus, said amplification being based on pair of oligonucleotides according to any of claims 1-5, said probe being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 12: AGAGGGCTGT CATGCAACTA GAGATGCTGT GGGTACTAACC, or the complementary sequence thereof, provided with a detectable label.

7. Oligonucleotide, according to claim 6, wherein the probe is constituted by a molecular beacon, preferably consisting of:
SEQ ID 13: [6-FAM]-ccatgggCTGTCATGCAACTAGAGATGCTGTcccatgg-[DabSyl].

8. Use of an oligonucleotides' pair, according to any of the claims 1-5, in a nucleic acid amplification reaction or as a probe for the detection of SARS nucleic acid in a sample.

9. Method for the detection of SARS nucleic acid in a sample wherein the sample is subjected to a nucleic acid amplification reaction using a pair of oligonucleotides according to any of the claims 1-5 and suitable amplification reagents and the presence of any amplified nucleic acid is detected.

10. Method according to claim 9, wherein the detection of any amplified nucleic acid is carried out by reacting the sample with an oligonucleotide according to claim 6 or 7 under suitable hybridization conditions and detecting the presence of the label in any hybrids formed between the amplified sequence and the probe.

11. Method according to claim 9, wherein the amplification technique used is a transcription based amplification technique, preferably the NASBA, and the first oligonucleotide is provided with a promoter sequence recognized by a DNA dependent RNA polymerase.

12. Test kit for the detection of SARS in a sample comprising:

- set of oligonucleotides according any of claims 1-5,
- an oligonucleotide comprising a nucleic acid sequence substantially complementary to at least part of the amplified nucleic acid sequence, provided with a detectable label, according to claim 6 or 7,
- suitable amplification reagents.

13. Test kit according to claim 12, wherein suitable amplification reagents enable a transcription based amplification technique, preferably the NASBA.

## ABSTRACT

Nucleic acid sequences that can be used as primers and probes in the amplification and detection of SARS

The present invention is related to nucleic acid sequences that can be used in the field of virus diagnostics, more specifically the diagnosis of infections with a novel human coronavirus causing Severe Acute Respiratory Syndrome (SARS).

With the present invention nucleotide sequences are provided that can be used as primers and probes in the amplification and detection of SARS nucleic acid. The oligonucleotide sequences provided with the present invention are located in the polymerase gene of the SARS Coronavirus genome. It has been found that, by using the sequences of the present invention in methods for the amplification and detection of nucleic acid a sensitive and specific detection of SARS can be obtained. The benefit of the sequences of the present invention primarily resides in the fact that so far no primer pairs or hybridization probes have been developed that would permit amplification of RNA by using a transcription based amplification technique, preferably the NASBA.

The oligonucleotide sequences according to the present invention are especially useful in methods for the amplification of nucleic acid.

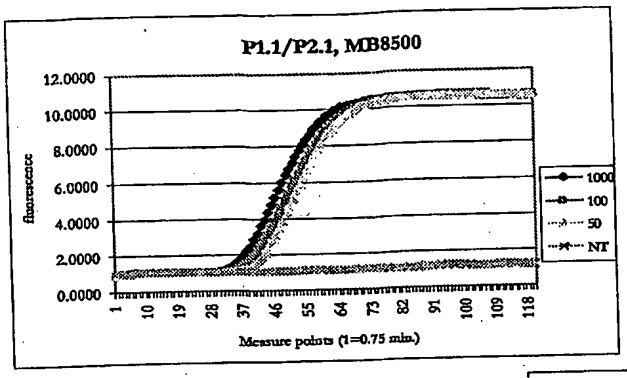


Fig. 1

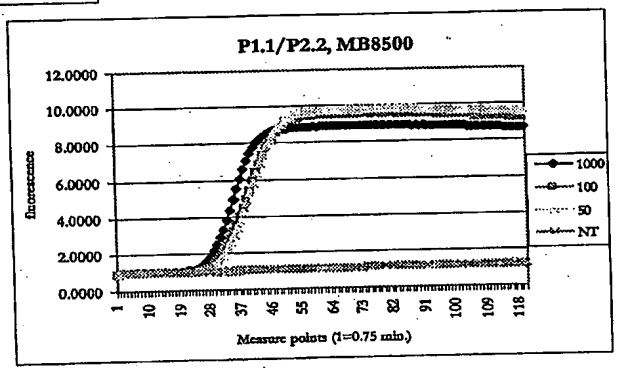


Fig. 2

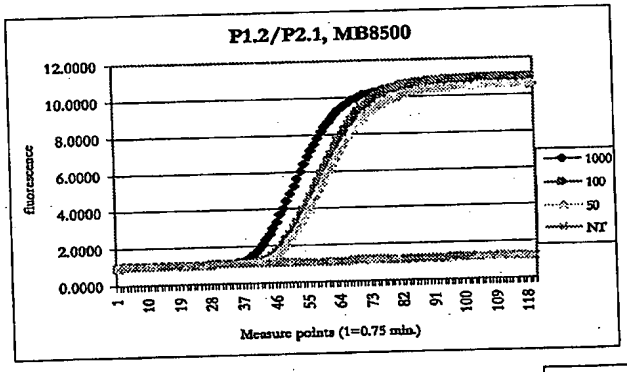


Fig. 3

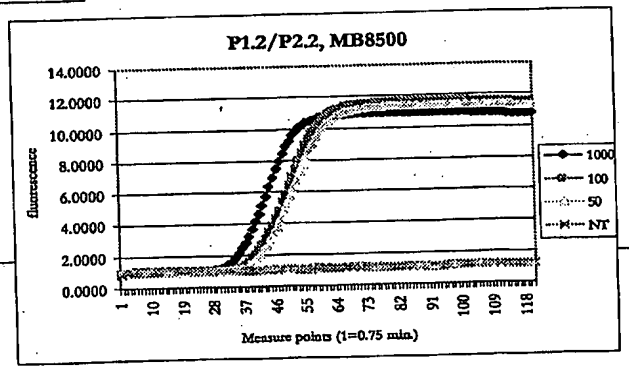


Fig. 4

# 2 / 2

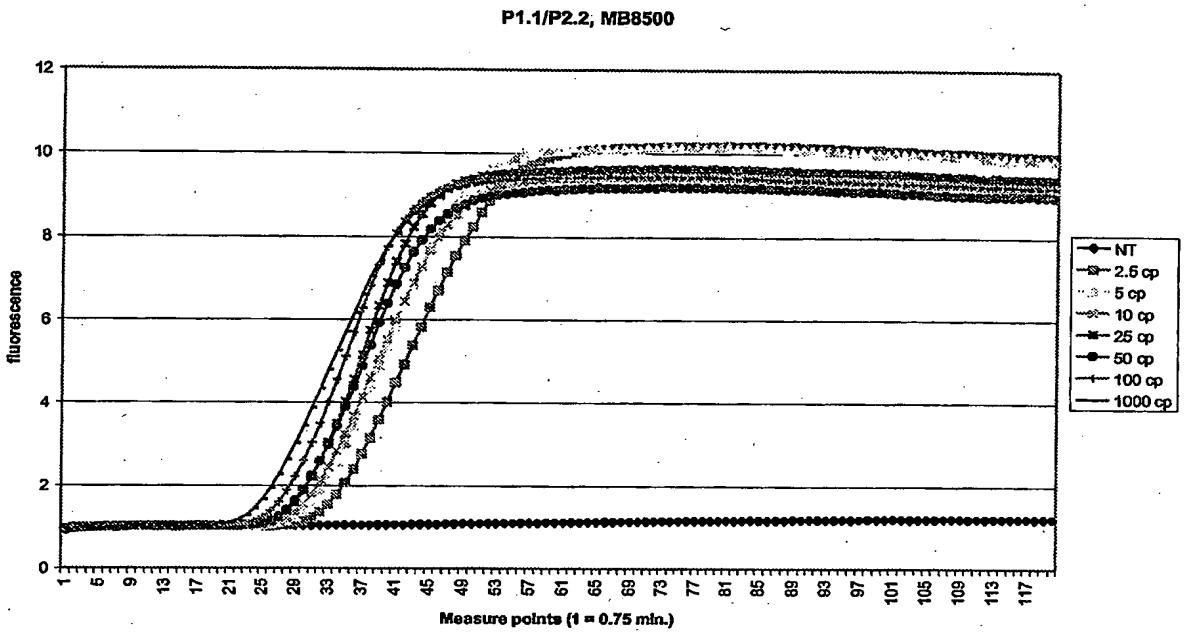
P1.1/P2.2; MB8500
fluorescence
Measure points (1 = 0.75 min.)
NT
2.5 cp
5 cp
10 cp
25 cp
50 cp
100 cp
1000 cp

Fig. 5